Electric Birefringence Study of the Purple Membrane of *Halobacterium halobium*

LEO D. KAHN and SHU-I TU, *Eastern Regional Research Center Philadelphia, Pennsylvania 19118*

Synopsis

An electric birefringence study was carried out on aqueous suspensions of the purple membrane of *Halobacterium halobium*. In addition to the characterization of both native and modified membrane samples, the dependence of electric birefringence on pH and ionic strength was also investigated. The results indicate that purple membrane shows electric birefringence at a field strength as low as 200 V/cm. The permanent dipole moment and polarizability ranged from 20,500 debyes and 1.01×10^{-14} cm^3 for a purple membrane concentration of 0.40 mg/mL to 41,000 debyes and 2.05×10^{-14} cm^3 for a concentration of 0.80 mg/mL. It was also found that removal of the retinyl group of bacteriorhodopsin substantially decreases but does not eliminate the electric birefringence of the membrane. The solubilization of the membrane by Triton X-100, however, completely abolishes the electric birefringence. These experiments indicate that there is an interaction between adjacent bacteriorhodopsin molecules within the purple membrane via the retinyl chromophore moiety that builds up the permanent dipole moment. They also suggest that there are two types of response when purple membrane suspensions are placed in an electric field. One is an alignment of the disk-shaped particles with the field. The other is a stacking of the particles following their alignment by the electric field, which is promoted by the induced dipole moment.

INTRODUCTION

The purple membrane of *Halobacterium halobium* consists of molecules of the protein, bacteriorhodopsin, imbedded transversely in a lipid membrane bilayer leaflet.[1] The bacteriorhodopsin has been shown to function as a proton pump through the action of light on its retinyl chromophore.[1] The proton electrochemical potential generated may serve as the driving force for the synthesis of adenosine triphosphate and other energy-demanding processes according to the chemiosmotic concept.[2] It is known that in the course of its function as a proton pump, bacteriorhodopsin goes through a photocycle involving five distinct intermediates characterized by the different states of its retinyl chromophore.[1] The chromophore is bound to the protein through a Schiff's base linkage.[3]

Included in the lipid portion of purple membrane are several unusual

* Agricultural Research Service, U.S. Department of Agriculture.

Note: Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

polar lipids,[4] which bear negative charges and are either glycerol ether derivatives[4] or sulfated polar lipids.[5]

Purple membrane has been shown to contain extended lattices made up of groups of trimeric units.[6] The monomeric subunit contains a single molecule of bacteriorhodopsin and its vicinal lipid molecules. The structure of bacteriorhodopsin is probably seven α-helices interlinked by salt bridges.[7] The protein has its N- and C-terminus regions exposed to the extracellular and cytoplasmic sides of the membrane, respectively.[8] The dimensions of isolated purple membrane particles have been reported as an approximate diameter of 500 nm and a thickness of 5 nm.[9] They may, therefore, be considered disks that, in an optical sense, are two-dimensional crystals having P_3 symmetry.[10] It has been shown that the chromophore has restricted mobility relative to the protein structure and that the bacteriorhodopsin is rigidly positioned.[11,12] Several investigators have carried out electric dichroism studies[9,13,14] indicating that purple membrane particles in suspension are aligned by an electric field. This observation suggested extending this work to the related technique of electric birefringence, which has been completely neglected in purple membrane investigations. Consequently, electric birefringence observations were carried out on native and modified preparations of purple membrane. Because the electric birefringence response was found to be a function of several solution parameters, the pH dependence and ionic strength dependence were investigated. Further studies were carried out after the purple membrane was modified by breaking the Schiff's base bond that attached the chromophore to the protein (bleaching); after the particles were broken down to monomeric units by detergent; and also after partial digestion by chymotrypsin. This research emphasizes the electric properties of purple membrane as elucidated by electric birefringence, rather than the physiological function of proton pumping.

If bacteriorhodopsin is kept in the dark at neutral pH and room temperature for several hours, its absorbance maximum shifts from 568 to 558 nm, which shows that a structural change has taken place.[1,3] Subsequent exposure to moderate light for just a few seconds restores the initial state. In this research it was assumed that the light-adapted state prevailed, since all operations (except monomerization) were carried out under daylight conditions.

MATERIALS AND METHODS

The isolation and purification of purple membrane from cultured *H. halobium* (R_1) followed a published procedure[15] as adopted in a previous publication.[16] SDS–acrylamide gel electrophoresis of the preparation revealed only one band with a molecular weight of 26,000.

The bleached membrane was prepared according to the procedure of Oesterheldt and Hess[17] with minor modifications. Purple membrane suspensions containing 3 mg bacteriorhodopsin in 2*M* hydroxylamine at

pH 6.8 in a temperature-controlled Gilson medical oxygen monitoring chamber were illuminated with a 750-W slide projector at 5°C until bleached. The bleached membrane was then washed three times by suspension in water and centrifuged. The final membrane pellet obtained by centrifugation at 48,000 × *g* was resuspended with proper buffer for electric birefringence measurements. On the addition of all-*trans* retinyl to bleached membrane (molar ratio of retinyl to apo-protein, 1.1:1), the characteristic absorption spectrum (Fig. 2 in Ref. 3) was recovered; this showed that more than 90% of the original material was recovered within 10 min at room temperatures.

Monomeric bacteriorhodopsin was prepared according to the method of Lind et al.[18] Purple membrane containing ~10 mg protein/mL in a solution of 10% Triton X-100 and 0.1*M* Tris-HCl, pH 7.0, was incubated in the dark for 12 h at room temperature. The solution was then centrifuged at 100,000 × *g* for 1 h to remove nondispersed membrane. The supernatant was then passed through a Sepharose 4B column equilibrated with 1% Triton X-100. This procedure effectively removed all the endogenous lipids normally associated with the protein. Chymotrypsin-treated purple membrane was prepared according to a published procedure[19] except that intact membrane particles were used. This treatment completely cleaved bacteriorhodopsin into C-1 (M_w ~ 19,000), and C-2 (M_w ~ 7000) fragments as determined by SDS–polyacrylamide gel electrophoresis.

A schematic diagram of the electric birefringence apparatus,[20] as well as details of the cell design,[21] have already been published. White light from a 100-W projection lamp was used, and the applied pulses were produced by a Cober pulse generator, model 606P.

The pH dependence of the electric birefringence of purple membrane was investigated by measuring a suspension of purple membrane in 0.01*M* K_2HPO_4 at a concentration of 0.55 mg/mL. Microliter amounts of 0.20*M* HCl were added sequentially to obtain the range of pH values.

To make certain that the observed response was not due to an aggregation initiated by a shift in pH, a light-scattering experiment was carried out. A fluorimeter was used because it provided an optical cell with entrance and exit beams set at a right angle. With both monochrometers set at 645 nm, scattered light was measured over the pH range of 4.99–8.16. The deviation in intensity of light scattered at 90°C never exceeded 4.5%; the maximum experimental error in the electric birefringence experiments was 6%.

The ionic strength dependence was determined using a series of purple membrane suspensions in phosphate buffer at pH 7.3 ± 0.1.

The rise in temperature that occurred in the electric birefringence cell because of the energy dissipated was calculated from the electrical parameters involved. This never exceeded 2°C, except when ionic strength dependence was investigated, as is noted further on.

The incidence of electrochromism in the purple membrane suspension

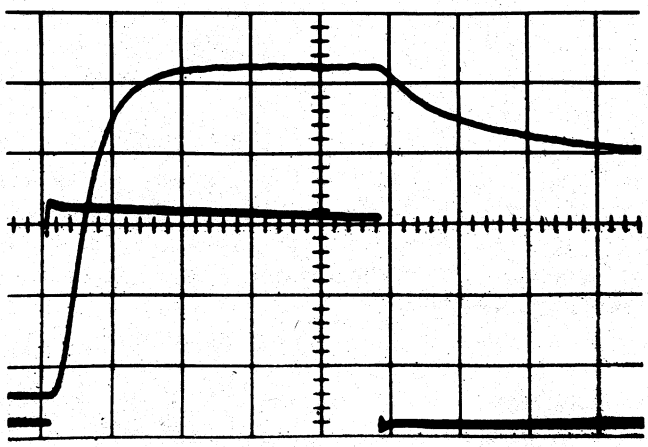

Fig. 1. Electric birefringence pattern for the purple membrane of *Halobacterium halobium* suspended in phosphate buffer at a concentration of 0.87 mg/mL, pH 5.69, and ionic strength of 0.005. The sweep is 0.50 ms/cm. The vertical scale for the pulse is 100 V/division applied across a gap of 0.210 cm. The vertical scale for the optic response is 1 mV/division. The birefringence at saturation is 44.6° of angle.

was investigated by removing the polarizing optical elements from the electric birefringence apparatus and recording optical absorbance changes when the field was applied. Absorbance changes were observed only at

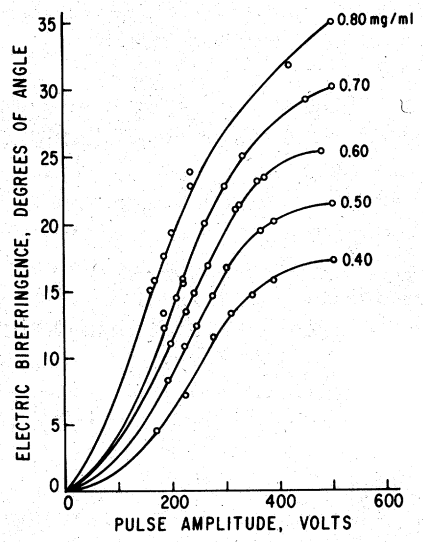

Fig. 2. Family of curves of electric birefringence of purple membrane at saturation expressed in degrees of angle versus pulse amplitude for indicated values of concentration (mg/mL). The cell gap was 0.210 cm, and the dispersion medium was phosphate buffer at pH 6.75 and ionic strength of 0.005.

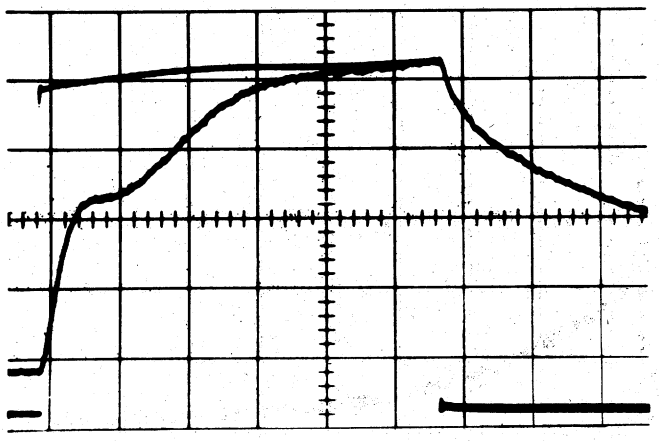

Fig. 3. Electric birefringence pattern for purple membrane suspended in phosphate buffer at a concentration of 0.20 mg/mL, pH 6.10, and ionic strength of 0.005. The sweep is 2.0 ms/division. The vertical scale for the pulse is 20 V/division and the cell gap was 0.210 cm. The vertical scale for the optic response is 0.20 mV/division. The electric birefringence at saturation is 6.0°.

field strengths greater than 10,000 V/cm. In this research, the field strength never exceeded 7200 V/cm.

RESULTS

Electric Birefringence and Dipole Moments

A typical electric birefringence pattern for purple membrane at a concentration of 0.87 mg/mL in phosphate buffer at pH 5.69 and ionic strength of 0.005 is shown in Fig. 1. In the course of the experiments described here, two unusual aspects of the birefringent response were observed. First, it was seen that very low values of field strength are capable of yielding a characteristic electric birefringence pattern. The pattern in Fig. 1 was made at a field strength of 1400 V/cm. However, good patterns were obtained with purple membrane when the field strength was as low as 200 V/cm. The unusual aspect of this becomes apparent when one considers that in the case of macromolecules previously studied by electric birefringence (tobacco mosaic virus, collagen), field strengths in the range of thousands of volts per cm were necessary to obtain a viable pattern. Kesthelyi has noted an equally sensitive response of purple membrane to an electric field in his electric dichroism measurements.[9]

In the second instance, it is noted in Fig. 1 that the decay of birefringence is very much slower than the buildup. This is another unusual observation, and it suggests that the hydrodynamics of the suspension have been altered by the electric field.

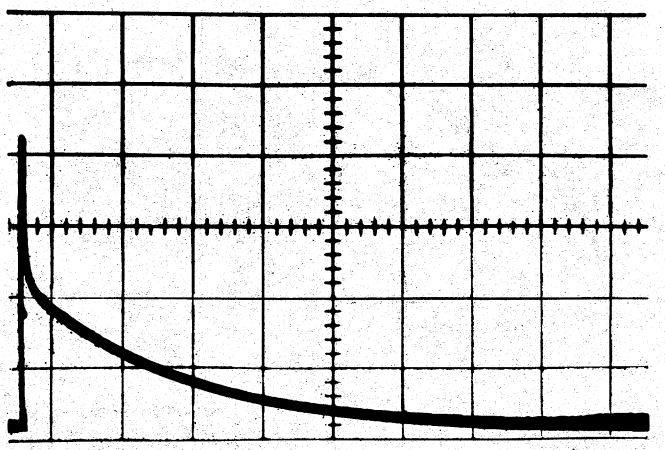

Fig. 4. Electric birefringence pattern for purple membrane with the time base expanded to emphasize the decay portion of the curve. The concentration of purple membrane was 0.80 mg/mL in phosphate buffer at pH 6.75 and an ionic strength of 0.005. The vertical scale is 1 mV/division, and the sweep is 50 ms/division. The width of the applied pulse was 2 ms. The field strength was 1900 V/cm, and the electric birefringence at saturation is 34°.

Figure 2 shows a family of curves of the electric birefringence at saturation for suspensions of purple membrane plotted in terms of concentration and pulse amplitude. When data of this sort are plotted as angle of birefringence versus the square of the field strength, they pass through the origin. The portion of the curve near the origin is usually straight, and this defines a Kerr region.[21] That, however, is not the case with purple membrane.

Permanent dipole moments and polarizabilities were calculated by the method of matching experimental data to theoretical curves, as outlined by Yoshioka and Watanabe.[22] The polarizability provides a measurement of the induced dipole moment, since the latter parameter is the product of polarizability and field strength. The step in this calculation that introduces the greatest degree of uncertainty is the extrapolation to zero field strength of the ratio: angle of birefringence/square of field strength. In the research reported, it was found that at low values of field strength this ratio follows the relation:

$$(n/E^2)_{E\to 0} = Ae^{-BE^2}$$

Here, n is the angle of birefringence, E the field strength, and A and B are constants for a given set of conditions. This fortuitous situation made it possible to determine an extrapolated value with ease.

The permanent dipole moment and polarizability of purple membrane increased with increasing concentration. These values ranged from 20,500 debyes and 1.01×10^{-14} cm^3 for a purple membrane concentration of 0.40

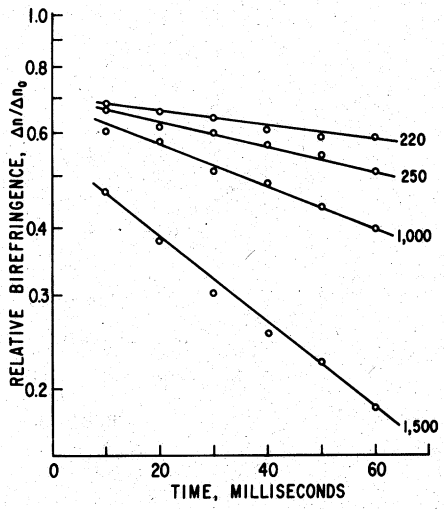

Fig. 5. Family of curves of the electric birefringence of purple membrane plotted as relative birefringence versus decay time for indicated values of applied electric pulse (V). The concentration of purple membrane was 0.80 mg/mL in phosphate buffer at pH 6.75 and an ionic strength of 0.005. The pulse width was 3.0 ms; the cell gap, 0.210 cm. The rotatory diffusion constants are: for 220-V amplitude, 0.52 s^{-1}; 250-V amplitude, 0.91 s^{-1}; 1000-V amplitude, 1.4 s^{-1}; 1500-V amplitude, 2.6 s^{-1}.

mg/mL to 41,000 debyes and 2.05×10^{-14} cm^3 for a concentration of 0.80 mg/mL.

Anomaly in Electric Birefringence Build-up Curve

When the electric birefringence experiment is carried out on purple membrane suspensions at very low values of field strength and/or concentration, the type of pattern shown in Fig. 3 appears. This pattern was made at a purple membrane concentration of 0.20 mg/mL and a field strength of 476 V/cm. It shows a shoulder at the leading edge of the electric birefringence build-up, indicating that build-up is a two-step process.

The Decay Curve

A closer examination of the decay curve is possible in Fig. 4, where the time base has been expanded to 50 ms/division. In this case, an aqueous solution of purple membrane at a concentration of 0.80 mg/mL was oriented by a field strength of 1900 V/cm for 2.0 ms. About 10 ms after the pulse appears, there is a break in the decay curve that separates the decay process into two stages. When this decay curve is replotted on semilogarithmic graph paper, the portion after the break appears as a straight line. This is shown in Figs. 5 and 6, where the break in the decay curve is made obvious

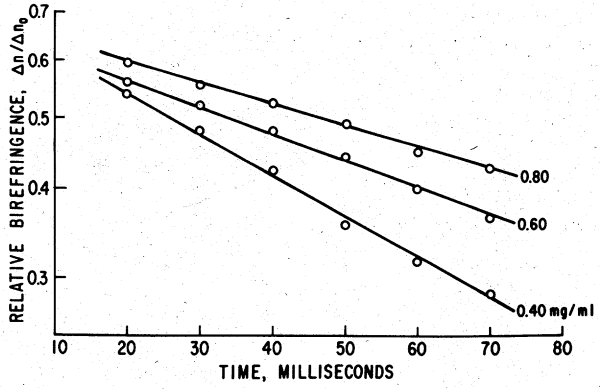

Fig. 6. Family of curves of the electric birefringence of purple membrane in phosphate buffer at pH 6.75 and an ionic strength of 0.005 plotted as relative birefringence versus decay time for indicated concentrations (mg/mL). The applied pulse was 600 V across a gap of 0.210 cm. The pulse width was 3.0 ms. The rotatory diffusion constants are: for 0.80 mg/mL, 1.2 s^{-1}; 0.60 mg/mL, 1.35 s^{-1}; 0.40 mg/mL, 2.0 s^{-1}.

by the fact that the plotted lines that must pass through the unit ordinate at zero time will do so only if there is curvature in the early (unplotted) part of the graph. The linear shape of the plotted lines indicates that in the region of decay beyond the break, the suspension is monodisperse.

Decay of birefringence is faster at high field strengths (as shown in Fig. 5) and also faster at low concentrations of purple membrane (Fig. 6).

Modified Purple Membrane

Bacteriorhodopsin in purple membrane shows a rather unusual CD spectrum in the absorption range of the retinyl chromophore.[23] As the result of regeneration experiments, Becher and Ebrey[24] concluded that the observed CD anomaly is due to a chromophore–chromophore interaction in the protein cluster. This exciton interaction may be eliminated by either removing the chromophore from the protein through photobleaching or by dispersing the membrane in a detergent.[24] The latter treatment generates protein monomers imbedded in detergent micelles.[25] Use of Triton X-100 as the detergent reduces the rate of photoisomerization (conversion of all-*trans* to 13-*cis* retinyl) in bacteriorhodopsin.[26] Monomeric bacteriorhodopsin in Triton X-100 micelles showed no birefringent response at field strengths up to 2900 V/cm.

The bleached purple membrane showed a marked reduction in electric birefringence and a faster decay than the native material. At a concentration of 0.67 mg/mL and at pH 6.73, and an ionic strength of 0.005, its permanent dipole moment was 14,000 debyes, and its polarizability was 7.0×10^{-15} cm^3, values about one-half those observed for native purple membrane under similar conditions.

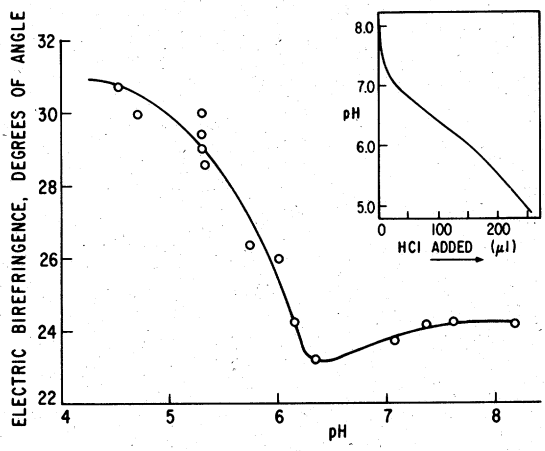

Fig. 7. Graph showing the pH dependence of the electric birefringence at saturation of purple membrane suspended in phosphate buffer at an ionic strength of 0.010. The field strength was 2380 V/cm; concentration of purple membrane, 0.55 mg/mL. The insert shows the titration curve for a suspension of purple membrane containing 6 mg bacteriorhodopsin in 10 m*M* KCl. This was titrated vs 8 m*M* HCl.

The electric birefringence pattern of the chymotrypsin-treated purple membrane showed no shoulder even at low concentrations and low field strengths. The electric birefringence was about half the values shown in Fig. 2 when measured under the same conditions.

The pH dependence of the electric birefringence of purple membrane is shown in Fig. 7, and it can be noted that there is a sharp minimum in the vicinity of pH 6.3. The inserted titration curve shows that there is no abrupt ionization point within the range of pH investigated. Tsuji and Neumann[27] have shown that in an electric field, there is a shift in the pK_a of purple membrane over the range of pH 6.0–7.5. A change in the pK_a of a molecule indicates a structural change, and in this instance, there may be a relation between these two observations. The ionic-strength dependence of the electric birefringence of purple membrane is shown in Fig. 8, for pH held to 7.3 ± 0.1.

DISCUSSION

The large loss of electric birefringence that occurs as the result of either bleaching or monomerization suggests that there is intraparticle coupling between bacteriorhodopsin molecules via the chromophore, and this coupling builds up the permanent dipole moment. Beyond this, the absence of a Kerr region, and the increase in both the permanent dipole moment and the polarizability with concentration of purple membrane tend to indicate a particle–particle interaction.

The response of purple membrane to an electric field appears to be a two-step process in both build-up and decay because of the shoulder on the

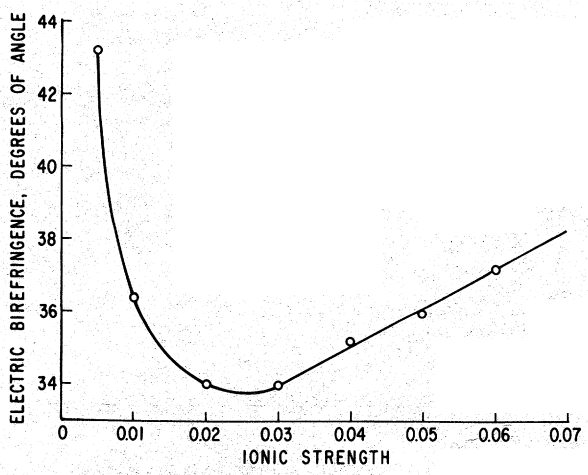

Fig. 8. Graph showing ionic-strength dependence of the electric birefringence at saturation of purple membrane in phosphate buffer at pH 7.3 ± 0.1. The field strength was 2300 V/cm; concentration, 0.80 mg/mL.

build-up curve and the break in the characteristic of the decay curve. It has been reported to be a two-step process by Shinar et al.,[28] who saw it as an alignment of chromophores within the membrane and a subsequent alignment of particles with the electric field. However, because of the low mobility of the chromophore within the membrane, an alternate scenario, which is purely hydrodynamic, and which is in keeping with the observations herein reported is offered.

The purple membrane particles are normally kept apart by Brownian motion. On application of the pulse, the electric field orients them, restricts their motion, and creates the induced dipole moment. In the latter action, it manipulates the charges so that the membrane becomes more strongly polarized and the electrostatic forces form a pseudoquaternary structure of laminar stacks with faces of opposite polarity facing each other. The stacking increases the permanent dipole moment by increasing the distance between the terminal charges and increases the polarizability by allowing more latitude for charge displacement. In the course of this process, counterions are forced out from between the particles, since both positive and negative charges are present on the membrane surfaces and these are in relatively close proximity. When the pulse is initiated, alignment begins and the shoulder of the build-up curve starts to form. When the induced dipole moment reaches a critical point, the stacking begins and the second step of the build-up appears. When the field is terminated, the induced dipole moment is lost and once separated, the particles continue in a normal decay.

It is probable that the reason a faster rate of decay in electric birefringence is observed at high field strengths is stronger interaction in stacking; this decreases the distance between adjacent particles so that there is greater repulsion when the field is removed. Decay is faster at low con-

centrations of purple membrane because of the smaller opportunity for stacking. The shoulder of the build-up curve is most prominent at low concentrations of purple membrane and at low field strength. This appears reasonable, since with a smaller population of particles, it will take a longer period of time to assemble a sufficient number in close enough proximity to initiate the stacking, and the lower the field strength, the lower the driving force forming the stacks. At high concentrations and high field strengths, the alignment stage extends into the stacking stage, the boundary between them becomes indistinct, and the shoulder is not apparent.

The combined knowledge that bleaching purple membrane by means of hydroxylamine breaks the bond between chromophore and protein at the Schiff's base linkage and that the bleached membrane displays a smaller electric birefringence is an indication that the high permanent dipole moment observed in the native material may be due to a concatenation between bacteriorhodopsin molecules on the same particle, and the coupling is probably through the chromophore moiety. The chromophore appears to be too small to have such a drastic influence on the electric birefringence by acting independently; but by acting as a link between bacteriorhodopsin molecules, it can influence dipole moments and polarizability.

Chymotrypsin treatment of purple membrane splits but one bond (between Phe-71 and Gly-72) of the bacteriorhodopsin peptide chain, which is located external to the membrane.[29] There are no charged groups on this portion of the peptide chain. This limited proteolytic treatment has no significant effect on the biological function of the protein.[30] That this treatment lowers the electric birefringence by one-half is evidence that integrity of the peptide chain is necessary for build-up of the permanent dipole moment. The loss of the shoulder of the electric birefringence build-up curve via chymotrypsin indicates that the integrity of this exposed fragment of bacteriorhodopsin is necessary for electric-field-induced stacking to occur.

Up to an ionic strength of 0.015, electric birefringence decreases with increasing electrolyte concentration (as shown in Fig. 8). This indicates an electrostatic effect. Beyond the ionic strength value of 0.015, it is quite likely that a denaturation due to heating supervenes. Since the electric birefringence of the purple membrane is a function of pH and ionic strength, it becomes apparent that electro-optical measurements of purple membrane must be carried out under controlled conditions.

While the source of the electric dipole of purple membrane has not been identified, it appears in this case that electrostatic properties can lead to elucidation of structure. This illustrates the usefulness of electric birefringence in pursuing this type of problem.

References

1. Stoeckenius, W., Lozier, R. H. & Bogomolni, R. A. (1979) *Biochem. Biophys. Acta* **505,** 215–278.

2. Mitchell, P. (1966) *Biol. Rev.* **41,** 445–501.

3. Stoeckenius, W. (1980) *Acc. Chem. Res.* **13,** 337–344.
4. Kushwaha, S. C., Kates, M. & Stoeckenius, W. (1976) *Biochem. Biophys. Acta* **426,** 703–710.
5. Kushwaha, S. C., Kates, M. & Martin, W. G. (1975) *Can. J. Biochem.* **53,** 284–292.
6. Henderson, R. (1975) *J. Mol. Biol.* **93,** 123–128.
7. Engleman, D. M., Henderson, R., McLachlan, A. D. & Wallace, B. A. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2023–2027.
8. Gerber, G. E., Gray, C. P., Wildenauer, D. & Khorana, H. G. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5426–5430.
9. Kesthelyi, L. (1980) *Biochem. Biophys. Acta* **598,** 429–436.
10. Henderson, R. & Unwin, P. (1975) *Nature* **257,** 28–32.
11. Hess, B., Korenstein, R. & Kuschnitz, D. (1978) *Collog. Ges. Biol. Chem.* **29,** 152–156.
12. Pasternak, C., Corda, D., Stieve, H. & Shinitsky, M. (1981) *Ann. NY Acad. Sci.* **366,** 265–273.
13. Tsuji, K. & Neumann, E. (1981) *Int. J. Biol. Macromol.* **3,** 231–242.
14. Neumann, E. & Tsuji, K. (1981) in *Molecular Electro-Optics*, Krause, S., Ed., Plenum, New York, pp. 241–267.
15. Osterheldt, D. & Stoeckenius, W. (1974) *Methods Enzymol.* **31,** 667–678.
16. Tu, S-I., Cavanaugh, J. R. & Hutchinson, H. (1982) *Biochem. Biophys. Res. Commun.* **106,** 23–29.
17. Osterheldt, D. & Hess, B. (1973) *Eur. J. Biochem.* **37,** 316–326.
18. Lind, C., Höjeberg, B. & Khorana, G. (1981) *Proc. Natl. Acad. Sci. USA* **256,** 8298–8305.
19. Gerber, G. E., Andregg, R. J., Herlihy, W. C., Grey, C. P., Breman, K. & Khorana, H. G. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 227–231.
20. Kahn, L. D. & Witnauer, L. P. (1969) *J. Am. Leather Chem. Assoc.* **64,** 12–18.
21. Kahn, L. D. (1972) *Methods Enzymol.* **26c,** 323–337.
22. Yoshioka, K. & Watanabe, H. (1969) in *Physical Principles and Techniques of Protein Chemistry*, Part A, Leach, S. J., Ed., pp. 355–367.
23. Becher, B. & Cassin, J. Y. (1975) *Biophys. Soc. Abstr.*, 65–66.
24. Becher, B. & Ebrey, T. G. (1976) *Biochem. Biophys. Res. Commun.* **69,** 1–6.
25. Dencher, N. A. & Heyn, M. P. (1978) *FEBS Lett.* **96,** 322–326.
26. Casadio, R. & Stoeckenius, W. (1980) *Biochemistry* **19,** 3374–3381.
27. Tsuji, K. & Neumann, E. (1981) *FEBS Lett.* **128,** 265–268.
28. Shinar, P., Druckman, S., Ottolenghi, M. & Korenstein, R. (1977) *Biophys. J.* **19,** 1–5.
29. Ovchinnikov, Y. A., Abdulaev, N. G., Feigina, M. Y., Kiselev, A. V. & Lobanov, N. A. (1979) *FEBS Lett.* **100,** 219–224.
30. Abdulaev, N. G., Feigina, M. Y., Kiselev, A. V., Ovchinnikov, Y. A., Drachev, L. A., Kaulen, A. D., Khitrina, L. V. & Skulachev, V. P. (1978) *FEBS Lett.* **90,** 190–194.